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Our research has had as its primary objective the investigation of the biodegradation processes involved in the interaction between microorganisms and polymers of interest to the Air Force, particularly polyurethane. We have grown polyurethane-degrading microorganisms from soil which had been contaminated with polyurethane-based paints for a number of years. Electrochemical impedence spectroscopy demonstrated that mixtures of microorganisms isolated from the contaminated soil yielded significant decomposition of the polymer within two months. Experiments with pure polyurethane showed that the microorganims utilized the polyurethane as a source of carbon and energy, rather than industrial contaminants. We showed that the adhesion of microorganisms to solid surfaces exerted a positive effect on the degradation process. The possibility of using bioreactors was considered. The ability of slow growing microorganisms to benefit from their attachment to solid surfaces because the growth rate is uncoupled from hydraulic retention time would permit degradation of polymers over long periods of time. This AASERT fellowship project supported a doctoral student, Jennifer Byrnes Brower, until the completion of her doctorate in February 1996.

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Principal Investigator	Professor Ralph Mitchell Harvard University Division of Engineering and Applied Sciences Cambridge, MA 02138

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During the entire period of this project
no proprietary research was carried out and no patents were applied for.

1. ABSTRACT

It is becoming increasingly apparent that many bacteria and fungi are capable of directly degrading complex organic polymeric materials. These include the polyurethanes, polyesters and epoxy compounds. Problems of delamination and blistering are often the result of microbial activity beneath the coating or between laminates. In addition, corrosion of the alloys beneath the coatings frequently occurs as a result of degradation of the polymers. Our research has had as its primary objective the investigation of the biodegradation processes involved in the interaction between microorganisms and polymers of interest to the Air Force.

Our research focused on polyurethanes. The Air Force uses large quantities of polyurethane-based coatings on its aircraft. These coatings are periodically removed, presenting a major problem for disposal.

We have used a variety of soils in order to isolate natural microorganisms capable of degrading polyurethane. In particular we have attempted to grow polyurethane-degrading microorganisms from soil which had been contaminated with polyurethane-based paints for a number of years. This approach was highly successful and resulted in our ability to isolate and grow a number of different bacteria with typical growth temperatures in the range of 15-26°C. In addition to bacteria, we grew a number of fungi which had the capacity to degrade polyurethane.

We carried out extensive electrochemical impedence studies to determine the effect of our microorganisms on the degradation process. Mixtures of microorganisms isolated from the contaminated soil yielded significant decomposition of the polymer within two months. We observed a dramatic decrease in resistance using electrochemical impedence spectroscopy with the consortium of microorganisms, indicating rapid degradation of the polyurethane.

We were concerned about the possibility that volatile contaminants added to the polyurethane during the manufacturing process might be responsible for the microbial activity. Experiments with pure, uncontaminated polyurethane showed that the microorganisms were indeed utilizing the polyurethane as a source of carbon and energy.

The organisms isolated in this study have the potential to be used to decontaminate polymeric coatings in storage and considered to be hazardous. The possibility of using microorganisms attached to surfaces in bioreactors was investigated in detail. We used soluble hazardous chemicals as our indicators of degradation. Our research showed that the adhesion of microorganisms to solid surfaces exerted a positive effect on the degradation process. The ability of slow growing microorganisms to benefit from their attachment to solid surfaces because the growth rate is uncoupled from hydraulic retention time would permit long-term retention in bioreactors, which would be particularly useful for treatment of stored hazardous chemicals.

This AASERT fellowship project supported a doctoral student, Jennifer Byrnes Brower, until

the completion of her doctorate in February 1996.

2. OBJECTIVES

The objective of this research was to investigate the biodegradation of polymeric materials that were important to the U.S. Air Force. The specific objective of our research was to determine the susceptibility of polymers to degradation by microorganisms isolated from natural environments. A further objective was to determine the possibility of developing bioreactors to degrade polymers and to determine methods of enhancing degradation of polymers considered to be hazardous and difficult to dispose.

3. EXPERIMENTAL METHODS

3.1 Experimental

Electrochemical impedance spectroscopy (EIS). EIS cells were constructed by gluing the polymers onto a 316 stainless steel coupon (50.0 x 50.0 mm) by a conductive silver epoxy (SPI Instrumental, West Chester, Pennsylvania). On the polymer film, a 30.0 mm long acrylic tube (I.D., 34.9 mm; O.D. 38.1 mm) was attached to the polymer-stainless steel

coupon by a mixture of Amercoat 90 resin (Ameron, Protective Coatings Group, Brea, California) and Epon 828 resin (Shell chemical Co., Houston, Texas) in a ratio of 4:1. After curing, the internal and external surfaces of the constructed EIS cells were thoroughly sterilized with 70% ethanol and left to dry in a laminar-flow sterile hood.

Our EIS consists of a Schlumberger 1250 frequency response analyzer combined with a Schlumberger 1286 electrochemical interface (Schlumberger Technologies -- Instruments Division, Billerica, Massachusetts). Z-plot software (Scribner Associates, Inc., Charlottesville, Virginia) was used to manipulate the system. During data acquisition, samples were potentiostatically held at their open circuit potential (OC)), and a sinusoidal perturbation of 20-50 mV was applied to the system. The impedance response was measured over a range of frequencies from 65 kHz to 1 mHz and spectra were recorded as a function of immersion time at ambient temperature and pressure. OCPs were monitored versus a saturated calomel electrode as a reference electrode of the trielectrode system. Platinum mesh was used in the EIS cell as a counter electrode, and the EIS cell as a working electrode. In all experiments, surface areas of the working electrode were 38.3 cm^2. Both Bode magnitude and phase angle plots as well as the Nyquist complex plane plots were used to provide information on increases in porosity, local defects and delamination.

Initially, a volume of 15.0 mL of sterile 0.2 M NaCl solution was added into the acrylic tube of the working electrode, and followed by 1.0 mL of a minimum salt solution. The salt solution consisted of (g per liter): K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $CaSO_4o2H_2O$, 0.05 g;

$MgSO_4 o 7H_2O$, 0.5 g; $FeSO_4 o 7H_2O$, 0.01 g; and $(NH_4)_2S0_4$, 1.0g. The measurement of the impedance responses was made after equilibration of the system. The uniformity of all prepared EIS cells was evaluated to determine the validity of using them in subsequent monitoring and to assign them to different treatments. The EIS cells used as working electrodes were divided into two groups. One set of the prepared EIS cells (four) were inoculated with 100 uL our microorganisms. Another set of EIS cells was kept sterile throughout the study by addition of 100 uL 0.1% sodium azide in the 0.2 M NaCl solution. At weekly or biweekly intervals, all EIS cells were analyzed for their impedance responses. The tri-electrode system was housed in a sterile laminar-flow hood. Aseptic procedures were used throughout to avoid contamination and cross contamination of the EIS cells. Both impedance and phase angle plots as well as Hyquist complex plane plots were used in the interpretation of polymer degradation. At the end of study the polymer film from inoculated and sterile EIS cells were taken and prepared for examination by scanning electron microscopy (SEM).

Scanning electron microscopy (SEM) sample preparation. Polymer film samples from the inoculated and the sterile control EIS cells were treated with 3% glutaraldehyde buffered with 0.2 M sodium cacodylate overnight. The solution was previously filtered through a 0.2-um-pore-size polycarbonate membrane filter (Gelman Science, Ann Arbor, Michigan). Film samples were then washed with 0.2 M Na cacodylate three times, fixed in 1% osmium tetroxide with 0.1 M Na cacodylate, and rinsed with 0.2 M Na cacodylate and deionized water three times for each treatment. The samples were dehydrated by immersing in an

ethanol-distilled water series of 40, 60, 70, 80, 85, 90, 95 and 100$ ethanol. Samples were stored in 100% ethanol and air-tight sealed glass vials before being critical point dried in liquid CO_2 (Smdri PVT-3B, Tousimis Research Co, Rockville, Maryland). Following drying, they were immediately coated with gold-palladium and viewed under an AMR 1000 Scanning Electron Microscope.

3.2 Enrichment cultures for microbial isolations.

An aerobic inorganic basal medium was utilized. The final pH was 7 and the medium was augmented with either liquid polyurethane or with solid form. In the latter case the polymer was coated on a glass surface and air dried. The soil samples were added to the growth media. The primary site for our inocula was a dump site near an auto repair shop where polymeric wastes had been disposed for approximately 50 years. Microorganisms were subcultured from the original enrichment cultures at regular intervals for a period of one year. Selected cultures were diluted 100 fold in sterile distilled water and subcultured a number of times in order to obtain purified isolates.

Identification.

In order to identify the bacteria, organisms were subcultured to nutrient auger. The BIOLOG system was used for identification of active bacteria. Fungal identification was determined using morphological characteristics.

4. RESULTS

In the initial phase of this research we attempted to isolate microorganisms capable of degrading polymers of interest to the U.S. Air Force. We studied polyurethane extensively. We isolated a number of bacteria and fungi from soil which had been contaminated for a long period of time with polyurethane-based paints. The microorganisms grew well on growth media in which the sole carbon source was polyurethane. The typical growth temperature was in the range of 15-26°C.

We used the BIOLOG system to identify the polyurethane-degrading bacteria isolated from contaminated soil. The predominant bacterium was Pseudomonas aeruginosa. However a number of other unidentified bacteria were capable of degrading polyurethane to a lesser degree. In addition to bacteria a number of fungi were identified. The dominant fungus belonged to the species Penicillium aspergilloides.

We carried out extensive electrochemical impedence studies in order to determine the effect of these microorganisms on polyurethane degradation. No degradation was detected in our samples maintained under sterile conditions. In contrast, mixtures of microorganisms isolated from contaminated soil yielded significant degradation of the polyurethane within two months, as determined by electrochemical impedence spectroscopy. Our data showed that the BODE plot changed in the samples inoculated with the mixed population of

bacteria. A dramatic decrease in resistance was observed following two months of incubation with the consortium of microorganisms, indicating that the polymer had been significantly degraded.

In contrast, our electrochemical impedence studies did not indicate any biodegradation of the polyurethane with either pure cultures of the fungus Penicillium aspergilloides or the bacterium Pseudomonas aeruginosa. No significant changes in the BODE plot were observed over two months with any of our other isolates, when inoculated alone to the polymer, indicating that a consortium of microorganisms is required for biodegradation to occur.

We were concerned about the possibility that volatile contaminants added to the polyurethane during the manufacturing process might be responsible for the microbial activity. Extensive studies were undertaken to decontaminate the polymer. In a typical experiment the polyurethane was placed in an oven at 50°C overnight in order to remove the volatile components. Cultures of the active consortium of bacteria were inoculated directly onto the surface of the cooled polymer. Excellent growth was observed within four to seven days. In similar experiments in which liquid polyurethane was mixed with growth medium the microbial consortium grew well on the polyurethane. It appears that these microorganisms are indeed responsible for the degradation of the polymer and that growth is not dependant on contaminants.

The effect of our microbial consortium on the polymer was studied in more detail using scanning electron microscopy. In this study a thin film of polyurethane was placed on agar and incubated for a month. The polyurethane was peeled off and a small section was cut out and examined using the scanning electron microscope. The microscopic study revealed a large amount of pitting in the areas in which the microorganisms were growing. When the surface was washed to clean the bacteria we observed significant polymer degradation. In a parallel experiment in which uninoculated polyurethane was incubated in the same growth medium no pitting or degradation was detected.

The effect of surfaces on the biodegradation potential of the microorganisms was examined in detail in this project. Surfaces have been shown to stimulate bacterial growth and metabolism. In our research we used polychloroethylene as our model chemical and a surface composed of granular activated carbon. Our consortium was shown to attach to granular activated carbon by both electron microscopy and epiflourescent light microscopy. We found that the microorganisms grew to a high density when they were attached to surfaces. When stress in the form of high temperatures was experienced, only a fraction of the exposed microbial population died. Thick biofilms were observed on all surfaces. Biodegradation at temperatures as low as 10°C was observed in our bioreactors. In addition to temperature shock, the reactor showed resistance to acids, drying, and low pH.

In our study the possibility that microorganisms adhering to surfaces might be used for bioremediation was considered. Our data showed that the consortium of microorganisms

which we had isolated adheres significantly to surfaces. This adhesion permits the microflora to continue metabolic activity over long periods of time. This is crucial in a bioremediation process where the rate of degradation is slow. It is particularly applicable to treatment of stored hazardous chemicals.

We have considered issues related to the integration of microbiological processes with engineering design in order to address the question of bioremediation by adherent microorganisms. Effective bioremediation of stored hazardous chemicals requires the ability to predict and control the adhesion of microorganisms and the metabolic activities under conditions of slow growth. The possibility of growing biofilms of microorganisms on contaminant polymers, or soluble chemicals sorbed to activated carbon so that the microorganisms are compartmentalized from the water, was investigated. We assumed that the degradation process is a result of sequential activities of a number of microorganisms in the consortium. Because the process is slow, the retention time on the surface was much higher than the hydraulic residence time. The complex interactions between microorganisms, surface, and aqueous phase need to be optimized before a prototype bioreactor can be built.

Our research project showed that polymers of importance to the Air Force can be degraded using microbial processes. Our investigation indicated that bioreactors could be constructed in which a range of hazardous chemicals are capable of being degraded. It may be necessary to incubate these surface-chemical-microorganism mixtures in bioreactors for long periods of time. Reaction design will require further microbiological research together with studies

of critical process parameters by environmental engineers. Furthermore, the reliability and stability of the biodegradation process needs to be determined.

This AASERT project supported the research of a doctoral student, Jennifer Byrnes Brower. Her doctoral thesis, The Effects of Surfaces on the Biodegradation of Pollutants, is available from the principal investigator.